呼吸筋不全の基礎的研究:低濃度酸素ガス吸入の影響

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研究組織

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研究発表

⑴学会誌等　（①進藤千代彦：呼吸筋疲労とは、（肺機能検査法と評価）、呼吸、１６（１）、３９－４４、１９９７；　②進藤千代彦：吸入補助システムの効率と効果、アレルギーの領域、４（８）、４６－５１、１９９７；　③C. Shindoh, D. Wu, Y. Ohuchi, H. Kurosawa, Y. Kikuchi, W. Hida, K. Shirato: Effects of L-NAME and L-arginine on diaphragm contraction in a septic animal model. Comp. Biochem. Physiol., 119A (1), 219-224, 1998；　④A. Taneda, C. Shindoh, Y. Ohuchi, K. Shirato: Protective effects of interleukin-10 on diaphragm muscle in a septic animal model. Tohoku J. Exp. Med. 180 (1), 45-54, 1998；　⑤C. Shindoh, D. Katayose, K. Shirato: Effects of interferon-α and -γ on diaphragm muscle in rats. Bull. Coll. Med. Sci. Tohoku Univ. 8 (1), 31-42, 1999)

⑵口頭発表　（①○進藤千代彦、片寄大、呉徳男、白土邦男：低濃度酸素吸入による横隔膜筋収縮力の変化。１９９７、４　第３７回日本胸部疾患学会総会（横浜）；　②○C. Shindoh, D. Katayose, D. Wu, and K. Shirato: Effects of Continuous Hypoxic Gas Inhalation on Diaphragm Muscle Contraction in Rats. American Thoracic Society (San Francisco), 1997 (5)；　③○C. Shindoh, M. Ichinose, G. Tamura, T. Takishima, and K. Shirato: Effects of anti-TNF-α antibody inhalation on endotoxin induced diaphragm dysfunction. The 11th Congress "International Society for Aerosols in Medicine" (Sendai), 1997(9)；　④○進藤千代彦、片寄大、白土邦男：Interleukin-8 の横隔膜筋収縮力への影響。１９９８、３　第３８回日本呼吸器学会総会（熊本）；　⑤○C. Shindoh, D. Katayose, and K. Shirato: Effects of Interleukin-8 on Diaphragm Muscle Contraction in Rats. American Thoracic Society (Chicago), 1998 (4))

⑶出版物　該当なし

研究成果

(1) 実験装置の作製について…本研究の実験装置は今回設備品に申請しているサーマル式ペンオシログラフの購入とともに、organ bath を作製し、測定系を完成させた。この装置をもちいて、課題である低酸素ガス吸入による横隔膜筋への影響に関する実験を実施した。

(2) 横隔膜筋収縮特性、筋線維の変化について…F_{IO_2} １０％の低濃度酸素ガスをラットの飼育チャンバーに持続的に流入させ、３日、１、２、３週間後に、横隔膜筋筋小片を作製し収縮特性を評価した。１、２週後に張力－周波数曲線が最も低下し、収縮は slow 化し、繰り返し刺激に対して疲労抵抗性に変化した。３週後にはいずれもコントロール値に戻る傾向を示し、低酸素状態での適応反応と思われる反応が見られた。筋線維の変化は ATPase 染色にて評価した。３日後に単位面積あたりの遅筋線維の速筋線維に対する比率が最も多くなり、遅筋線維優位に変化したが、次第にこの比率はコントロール値に復帰した。

(3) Hemo oxygenase (HO)、NO synthase (NOS)発現の検討…HO-1 は、第１日目に有為の発現がみとめたが、その後は減少した。HO はスーパーオキサイドに対して防御的な作用を持つ。又 iNOS は第１日目に低下したが、その後増加したのに対して、eNOS は全期間にわたって増加していた。

(4) 以上の結果から、低酸素負荷の早期にスーパーオキサイドを防御する HO-1 が増加、iNOS は減少し、これらが防御機構として働いていることが判明した。その後継続的な eNOS が見られ適応反応に関与している可能性が推察された。横隔膜筋はその発生張力の減少が呼吸筋不全に関係するが、本研究により、低酸素状態における横隔膜筋収縮力や筋線維の変化の機序として、HO-1, iNOS, eNOS 発現とが密接な関係にあることが結論づけられた。

参考文献

(1)当該論文

(2)発表論文その１

(3)発表論文その２

(4)発表論文その３

Effects of Continuous Hypoxia on Diaphragm Muscle Contraction and Fibers in Rats

by

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Key words: force-frequency curves; twitch kinetics; muscle fatigue

Abstract

Since hypoxemia is frequently observed in patients with respiratory diseases, we examined whether continuous hypoxic gas inhalation affects the diaphragm contractile properties in rat. Hypoxic gas ($FIO_2 \cong 0.10$) was produced by two reversely connected oxygen enrichers and was continuously fed into the breeding chamber. At 3, 7, 14, and 21 days of hypoxia (n = 6, respectively), we dissected the diaphragm muscle under light anesthesia, made muscle strips, and measured force-frequency curves, twitch kinetics and fatigability *in vitro*, and muscle fiber compositions by ATPase staining. At 7 days of hypoxia, the force-frequency curves decreased to 1.53 ± 0.07 kg/cm^2, both contraction time and half relaxation time elongated to 85 ± 5.0 and 93 ± 3.1 msec, respectively, and fatigability increased to 18 ± 1.3%. However, at 21 days, these parameters returned to near control values (1.75 ± 0.07 kg/cm^2, 67 ± 1.8 msec, 76 ± 4.7 msec, and 15 ± 0.9 %, respectively). At 3 days, type I (slow twitch) muscle fiber increased to 40.3 ± 2.2 %, and type II (fast twitch) decreased to 59.7 ± 2.2 %; at 21 days, however, these values also returned (32.1 ± 1.9 %, 67.9 ± 1.9 %, respectively) to near control values. From these results, we conclude that the diaphragm muscle in the early phase of hypoxia decreases force-frequency curves and slows contraction with corresponding fiber changes; however, these changes are reversed and a non-hypoxic state similar to the control is seen at 21 days. It is suggested that the diaphragm muscle has the ability to adapt to hypoxia.

Introduction

It is well known that hypoxia induces a decrease of skeletal muscle tension and enhances muscle fatigue (14). J. Jardim et al. reported that the effect of low oxygen breathing ($FIO_2 = 0.13$) on inspiratory muscle fatigue resulted in a shorter endurance time, a faster rate in the shift of the electromyographic power spectrum, and a greater rate of increase in blood lactate concentrations during inspiratory resistive breathing in normal subjects (13). In addition, exhaustive exercise during hypoxia ($FIO_2 = 0.12$) caused marked hyperventilation and reduced arterial O_2 content; glycogen fell in the plantaris (20% of control) and in the diaphragm (58%), the sparing effect of which is due primarily to glucose-6-phosphatase inhibition of glycogen phosphorylase in the diaphragm muscle (8). Furthermore, S. A. Esau reported that hypercapnic acidosis had a greater negative inotropic effect on the diaphragm muscle than did hypoxia alone, and made the muscle more susceptible to fatigue *in vitro* (7). Because the impaired endurance performance of muscles during physical exercise is a well-recognized response to conditions of acute normobaric or hypobaric hypoxia, it is considered to be closely related to the reduction in maximal aerobic power due to arterial hypoxemia (9).

However, these findings were concerned with relatively acute hypoxia of short duration, therefore, the effects of longer continuous hypoxia on the diaphragm contractile properties have not been well elucidated. Moreover, to our knowledge, diaphragm muscle fiber composition has not been examined under either acute or long hypoxia. In the present study, therefore, we examined whether the diaphragm muscle contractile properties and the compositions of type I (slow-twitch) and type II (fast-twitch) muscle fibers change during 21 days of hypoxia.

Methods and Materials

Animal preparation

Experiments were performed using 30 Wistar rats weighing 250-320 g (Charles River Japan, Kanagawa, Japan). The control group ($n = 6$) was loaded with ambient atmospheric gas ($FIO_2 = 0.21$), and the hypoxic gas inhalation group (total $n = 24$) was loaded with a hypoxic gas ($FIO_2 \cong 0.10$). The hypoxic gas was produced by feeding the exhaust gas from the first

oxygen enricher into the inlet of the second oxygen enricher; the oxygen fraction of the exhaust gas from the second oxygen enricher was approximately 10% ($FIO_2 \cong 0.10$). The hypoxic gas was continuously fed into breeding cages covered with translucent plastic sheets. The animals of each group were caged, isolated for the duration of the experiment, and maintained on a 12:12-h light-dark cycle at ambient temperature (23 - 25°C). We performed two kinds of measurement: (I) diaphragm muscle contractile properties were measured *in vitro* in the control group (n = 6) and in the hypoxic gas inhalation group at 3, 7, 14, and 21 days (n = 6 each), (II) muscle samples taken from the control group and the hypoxic gas inhalation group at 3, 7, 21 days were studied histologically by ATPase staining. Written approval was obtained from the Tohoku University Animal Facility.

Diaphragm muscle contractile measurements

The measurements of diaphragm muscle contractility were performed as previously reported (19). Briefly, two muscle strips (3-4 mm wide) were dissected from the right and left hemidiaphragm under diethyl ether anesthesia and mounted in separate organ baths containing Krebs-Henseleit solution oxygenated with a 95% O_2 - 5% C O_2 gas mixture (23.5 ± 0.5°C, pH 7.40 ± 0.05). The composition of the aerated Krebs-Henseleit solution in mEq/L was as follows: Na^+, 153.8; K^+, 5.0; Ca^{2+}, 5.0; Mg^{2+}, 2.0; Cl^-, 145.0; HCO_3^-, 15.0; HPO_4^{2-}, 1.9; SO_4^{2-}, 2.0; glucose, 110 mg%; 10 μM d-tubocurarine; regular crystalline zinc insulin, 50 U/L. Both muscle strips were simultaneously stimulated with supramaximal currents (i.e., 1.2 to 1.5 times the current required to elicit maximal twitch tension, 200-250 mA, pulses of 0.2 msec duration) by a constant current stimulus isolation unit (SS-302J, Nihon Kohden) driven by a stimulator (SEN-3201, Nihon Kohden). The elicited tensions were measured by a force transducer (UL-100GR, Minebea Co.). The length of each muscle strip was changed by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm), and measured with a micrometer in close proximity to the muscle. The optimal length of the muscle (Lo) was defined as the muscle length at which twitch tension development was maximal, and this Lo was maintained in the following measurements.

The diaphragm force-frequency relationship was assessed by sequentially stimulating muscles at 1, 10, 20, 30, 50, 70, and 100 Hz. Each stimulus train was applied for approximately 1 sec, and adjacent trains were applied at intervals of approximately 10 sec. The tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K, San-ei, Tokyo). The force-frequency curves obtained from the groups studied were displayed as elicited tensions (kg/cm^2) on the Y-axis and stimulating frequencies on the X-axis.

Twitch contraction was elicited by single pulse stimulation (0.2 msec), and the trace of the twitch contraction was recorded at high speed (10 cm/sec). The twitch kinetics were assessed by (I) twitch tension (peak tension of twitch contraction, kg/cm^2), (II) contraction time (the time required to develop peak tension, msec), and (III) half relaxation time (the time required for peak tension to fall by 50%, msec) during a single muscle contraction.

Muscle fatigability was then assessed by examining the rate of fall of tension over 5 min of rhythmic contraction. Rhythmic contraction was induced by applying trains of 20 Hz stimuli (train duration, 0.33 s; rest duration, 0.66 s; train:rest ratios, 1:2) at a 60 train/min rate. Muscle fatigability was expressed as a percentage of the final remaining tension (%) from the initial tension. After completion of this protocol, the muscle strip was removed from the bath and weighed.

Then, muscle strips were adjusted to Lo and fixed with pins on a cork plate. Samples were immediately embedded in mounting medium (OCT compound, Miles, Elkhart, IN), immersed in isopentane (Wako Pure Chemical Industries Ltd., Osaka, Japan) that had been cooled in liquid nitrogen, and stored in a refrigerator (-80°C) to await further analysis.

Adenosine triphosphatase (ATPase) stain

Myofibrillar adenosine triphosphatase (ATPase) staining of the diaphragm was performed according to the method of Dubowitz and Brooke (5). Diaphragm sample tissues were sectioned to 10 μm with a cryostat (BRIGHT Instrument, Huntingdon, UK) kept at -20°C. On the basis of their staining reactions for myofibrillar ATPase, after alkaline preincubation (pH 10.4), muscle fibers were classified as either type I or type II. Unstained fibers were classified as type I (high oxidative slow-twitch), and stained fibers were classified

as type II (both high oxidative fast-twitch and low oxidative fast-twitch). Fiber cross-sectional areas were measured by digitizing with a computerized image-processing system (PIAS Co., Tokyo, Japan). The area was determined from the number of pixels within the outlined borders, with each pixel having a width of 0.125 mm. Fiber type proportions and cross-sectional areas (CSA) were determined from a sample of 350-400 fibers using several sections of each muscle. Fiber CSA (×20) were determined from the number of pixels within the outlined borders, with each pixel having an area of 0.676 μm^2 at ×20 magnification.

Data Analysis

The strip muscle cross-sectional area was calculated by dividing muscle mass by the product of strip muscle length and muscle density (1.06 g/cm^3), and tension was calculated as force per unit area (kg/cm^2) (4). The mean values for each frequency in force-frequency curves, twitch kinetics and fatigability were compared by Student's *t* -test. All data are presented as means ± SE. Data with a *p* value of less than 0.05 were considered statistically significant.

Results

Changes of muscle contractile properties

Figure 1 shows the mean force-frequency curves of the control group and those induced by hypoxia from 3 to 21 days. As for comparisons of the tensions at corresponding frequencies, the tensions in lower ranges (1 and 10 Hz) at 7 and 14 days were significantly increased, while those in higher ranges (70 to 100 Hz) were significantly decreased from those of the control force-frequency curve († $p < 0.01$, ‡ $p < 0.001$, respectively). These changes were caused by leftward shift of force-frequency curves during the early phase of hypoxia (i.e., 3 to 7 days). At 21 days, the tensions in the higher ranges had recovered to levels intermediate between 7 days and the control, and the tensions in the lower ranges had decreased to near control values.

Figure 2 summarizes the mean changes of twitch kinetics and fatigability in the control and hypoxia groups. In terms of twitch tension (A), the twitch tensions during hypoxic gas

inhalation maximally increased at 3 days ($p < 0.001$), then decreased by 21 days, although each twitch tension was significantly larger than the control value ($p < 0.01$). As for contraction times (B), they were maximally elongated by 7 days, then decreased to the control level by day 21 during hypoxic gas inhalation; each contraction time was significantly longer than the control value ($p < 0.001$). With regard to half relaxation time (C), it was also maximally elongated at 7 days ($p < 0.001$), then decreased by day 21 ($p < 0.05$) during hypoxic gas inhalation. The observed increases of both contraction time and half relaxation time at 7 days mean that the diaphragm muscle contracted more slowly than the control, then returned to the control level. Concerning fatigability (D), it was significantly increased at 7 and 14 days during hypoxic gas inhalation (both $p < 0.01$), while there was no significant change at 3 and 21 days. The increase of fatigability means that the diaphragm muscle became more fatigue resistant than in the control at 7 and 14 days, then returned to the control level.

Changes of muscle fiber composition

Figure 3 shows typical photographs of myofibrillar ATPase staining at alkaline pH of control (A), 3 days (B), 7 days (C), and 21 days (D) during hypoxia. In the alkaline pH, the unstained and stained fibers indicate type I (slow-twitch) and type II (fast-twitch) muscle fibers. At 3 days (B) and 7 days (C), because the number of unstained muscle fibers increased compared with the number in the control, it seems that the white area increased and the black area decreased, in the photograph.

Figure 4 shows the mean numbers (percentage) of type I and type II muscle fiber in the control and at 3, 7 and 21 days during hypoxia. The percentage of type I (slow-twitch) muscle fiber increased ($40.3 \pm 2.2\%$, $p < 0.001$) significantly from that of the control ($22.4 \pm 1.1\%$), then decreased at 7 and 21 days ($36.8 \pm 1.6\%$, $p < 0.001$; $32.1 \pm 1.9\%$, $p < 0.01$, respectively). Reciprocal changes in the percentages of type II were observed.

Figure 5 shows the mean changes of cross-sectional areas of type I and type II in the control and 3, 7 and 21 days during hypoxia. The cross-sectional area of type I muscle fibers was increased, but not significantly changed from that of the control. The cross-sectional area

of type II muscle fibers significantly increased at 3 and 7 days ($p < 0.001$), and at 21 days ($p < 0.01$) from that of the control.

Discussion

In the present study, continuous hypoxia ($FIO_2 \cong 0.10$) induced diaphragm muscle deterioration and a leftward shift of the force-frequency curves, an elongation of contraction and half relaxation times, and fatigue resistance accompanied by the increments of type I (slow-twitch) muscle fibers at 7 and 14 days. However, at 21 days of hypoxia, the force-frequency curves, twitch kinetics and muscle fiber composition returned toward control levels. These results suggest that the diaphragm muscle becomes to slow-twitch muscle fiber dominant muscle in the early phase of continuous hypoxia, and then shows an adaptive response to the hypoxia by regaining nearly normal contractile properties as a result of a return to normal muscle fiber composition.

Studies of the diaphragm during inspiration of elevated O_2 fractions have shown an increased resistance to fatigue and changes in ventilatory muscle recruitment allowing enhanced performance, measured as increased endurance time (16). Conversely, moderate hypoxia, induced by inspiration of 13% O_2, exacerbated inspiratory muscle fatigue as evidenced by decreased endurance time and earlier shifts in the electromyogram frequency spectrum (13). Three explanations are suggested as to why hypoxia may increase diaphragm fatigue during intense whole body endurance exercise: (I) increased work by the diaphragm, (II) decreased O_2 transport to the diaphragm, and (III) the influence of circulating metabolites from locomotor muscles working at a higher intensity in hypoxia (2). However, B. T. Ameredes et al. reported that fatigue of the inspiratory muscles of normal human subjects breathing 21% O_2 (normoxia), 13% O_2 (hypoxia), or 100% O_2 (hyperoxia) who performed repeated maximal inspiratory maneuvers on an isoflow system did not show significant differences among the three inspiration conditions (1). J. Yanos et al. examined whether respiratory muscle fatigue plays a role in respiratory arrest using a dog model. They reported that such fatigue may not be a major factor in respiratory arrest associated with inspiratory loading and hypoxia, and suggested that suppression of central drive may be important (22, 6). Thus, it can be seen that results to date

on the relationship of muscle fatigue and moderate hypoxia have been inconsistent, and that the diaphragm muscle, especially in human experiments, shows resistance to muscle fatigue.

On the other hand, several potential mechanisms or sites of failure may account for the hypoxic depression. In the adult diaphragm, hypoxia rapidly inhibits nerve conduction (12) and presynaptic transmitter release (15). Complemental studies have demonstrated that hypoxia depresses respiratory and nonrespiratory skeletal muscle as well as cardiac muscle contractility (17, 21). On the postsynaptic side, hypoxia causes a depolarization of resting membrane potential (20) and enhancement of miniature end-plate potential frequency (15, 12). A. R. Bazzy reported that neuromuscular transmission in the newborn diaphragm is more resistant to the effects of hypoxia than the older diaphragm and that the predominant effect of hypoxia is peripheral in the diaphragm muscle fibers, whereas in the older diaphragm the effect is before or at the neuromuscular junction (3). Although these previous studies concerning hypoxia focused on neuromuscular transmission and resting membrane potential of diaphragm muscle, it is possible that these changes of nerve conduction or cell membrane may trigger the changes in their fiber compositions.

In continuous hypoxia, an interesting finding is that the diaphragm muscle induced an increment in the number of type I (slow-twitch) muscle fibers. Because type I (slow-twitch) muscle fiber is more fatigue resistant than type II (fast-twitch) muscle fibers (4), it can be concluded that the changes of contractile properties were caused by the histological changes in muscle fibers. In a previous study, we reported a transient increment of type I (slow-twitch) muscle fibers in a denervated diaphragm (18). Although there are many differences in the experimental setups for investigating hypoxia and denervation, it has been generally observed that the contractile properties in type I dominant muscle show a decrease and leftward shift of force-frequency curves, an increase of contraction time and half relaxation time, and changes in fatigue resistance. It is clear that the number of type I dominant muscle fibers increases due to hypoxia, because it occurs in the early phase of hypoxia. However, the mechanisms resulting in these muscle fiber changes induced by continuous hypoxia are still unclear, and further analysis is needed.

Since the present study was performed at sea level (i.e., normobaric hypoxia), it is of particular interest to refer to experiments conducted at higher altitudes. S. H. Garner et al. studied the force of the ankle dorsiflexors during a 40-day simulated ascent of Mt. Everest in a hypobaric chamber; both electrically activated and maximal voluntary contractions were employed, and it was found that chronic altitude exposure did not appear to affect the maximal muscle force-generating capacity, but did have a mild effect on the susceptibility to fatigue during the exercise protocols. They concluded that the central motor drive becomes more precarious at higher altitudes and is associated with increased muscle fatigue at low excitation frequencies; the latter is the result, in part, of chronic hypoxia and occurs in the muscle fiber interior because no impairment in neuromuscular transmission could be demonstrated (11). C. S. Fulco et al. reported that maximal voluntary contractions of the rested adductor pollicis muscle are not impaired during or after acute (1 day) and chronic (13 days) exertion at high altitude (4,300 mm) (10). It is suggested that our results of a leftward shift of force-frequency curves are compatible with maintained maximal contractions at high altitudes and that our results on adaptation after transient changes may explain why several base camps are employed when mountain climbers attempt to reach the summit.

In conclusion, the diaphragm muscle under continuous hypoxia shows transient changes in contractile properties with changes in muscle fiber composition. The underlying mechanisms of these findings are unknown, but the observed phenomenon may be triggered by hypoxia resulting in a change in the composition of the diaphragm muscle fibers in the early phase, with a return to the control level in the late phase. If termed adaptation to continuous hypoxia, such adaptation of the diaphragm muscle may occur in the patients with chronic hypoxia, for example, chronic obstructive pulmonary disease (COPD), fibrosing lung disease (FLD), etc. Therefore, it is suggested that patients who suffer from COPD or FLD can tolerate chronic hypoxia for many years due to such adaptation.

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Figure legends

Fig. 1 Force frequency curves of control and hypoxia groups at 3, 7, 14 and 21 days. Symbols indicate significant differences at given frequencies compared with control (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$).

Fig. 2 Mean changes of twitch tensions (A), contraction time (B), half relaxation time (C), and fatigability (D) from the diaphragm of control and hypoxia groups at 3, 7, 14 and 21 days. Symbols indicate significant differences compared with control diaphragm (* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$).

Fig. 3 Typical photographs of control group (A), and hypoxia group at 3 days (B), at 7 days (C), and at 21 days (D). Magnification is ×200.

Fig. 4 Changes of numbers (percentage) of type I and type II muscle fibers. Symbols indicate significant differences compared with control diaphragm († $p < 0.01$, ‡ $p < 0.001$).

Fig. 5 Changes of cross-sectional areas of type I and type II muscle fibers. Symbols indicate significant differences compared with control diaphragm (‡ $p < 0.001$).

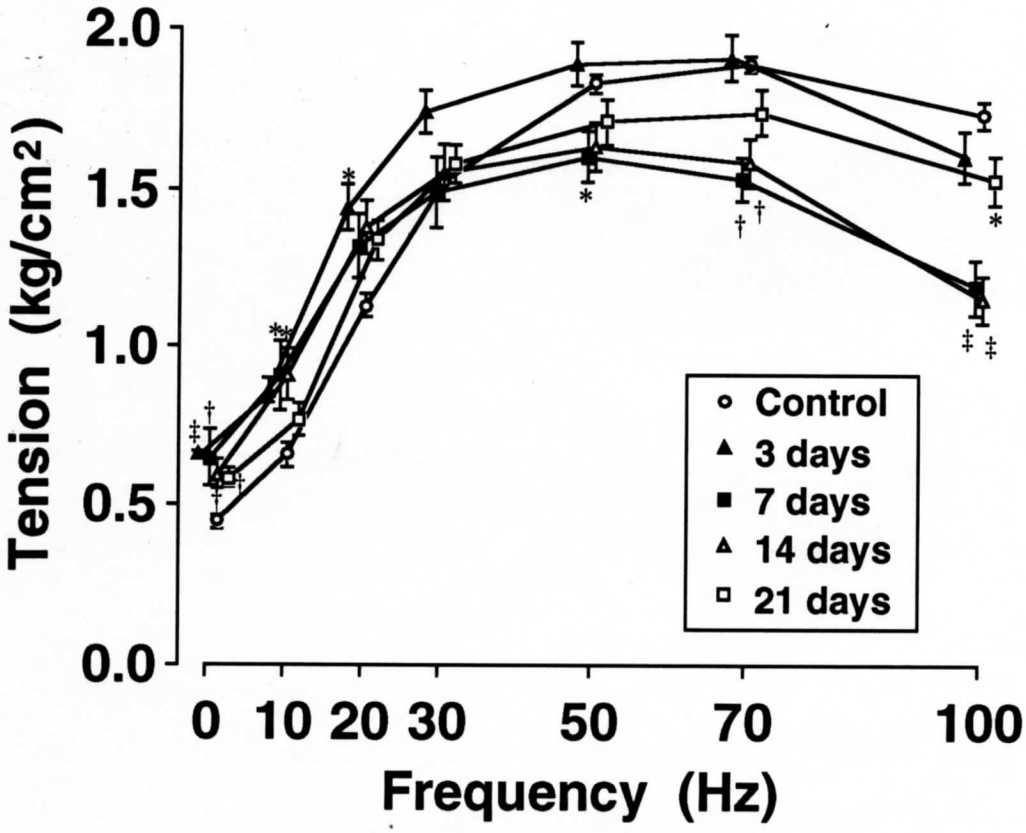
2.0
1.5
1.0
0.5
0.0
Tension (kg/cm²)
0 10 20 30 50 70 100
Frequency (Hz)
Control
3 days
7 days
14 days
21 days

Fig. 1

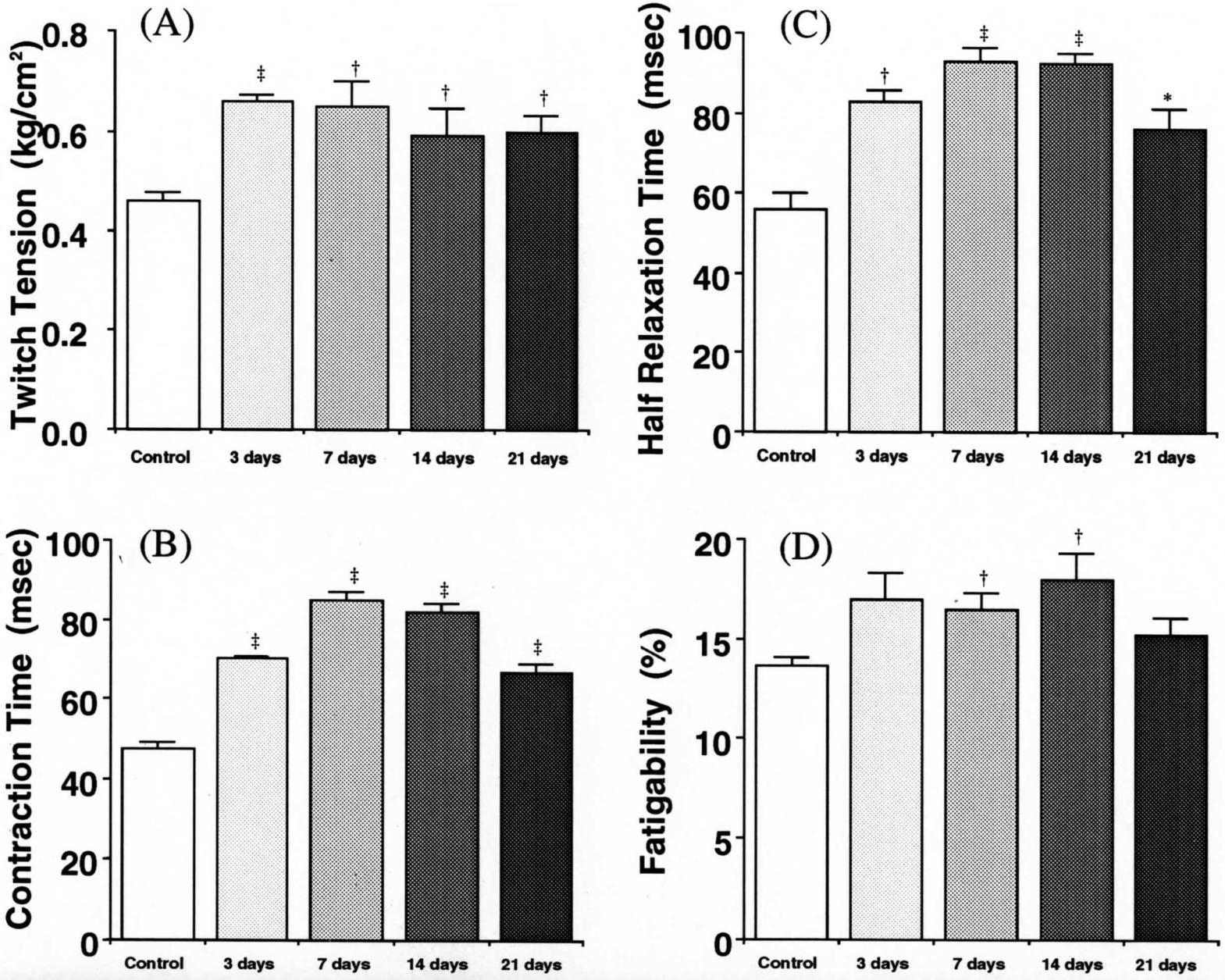
(A)
Twitch Tension (kg/cm²)
0.0
0.2
0.4
0.6
0.8
‡
†
†
†
Control
3 days
7 days
14 days
21 days
(C)
Half Relaxation Time (msec)
0
20
40
60
80
100
†
‡
‡
*
Control
3 days
7 days
14 days
21 days
(B)
Contraction Time (msec)
0
20
40
60
80
100
‡
‡
‡
Control
3 days
7 days
14 days
21 days
(D)
Fatigability (%)
0
5
10
15
20
†
†
Control
3 days
7 days
14 days
21 days

Fig. 2

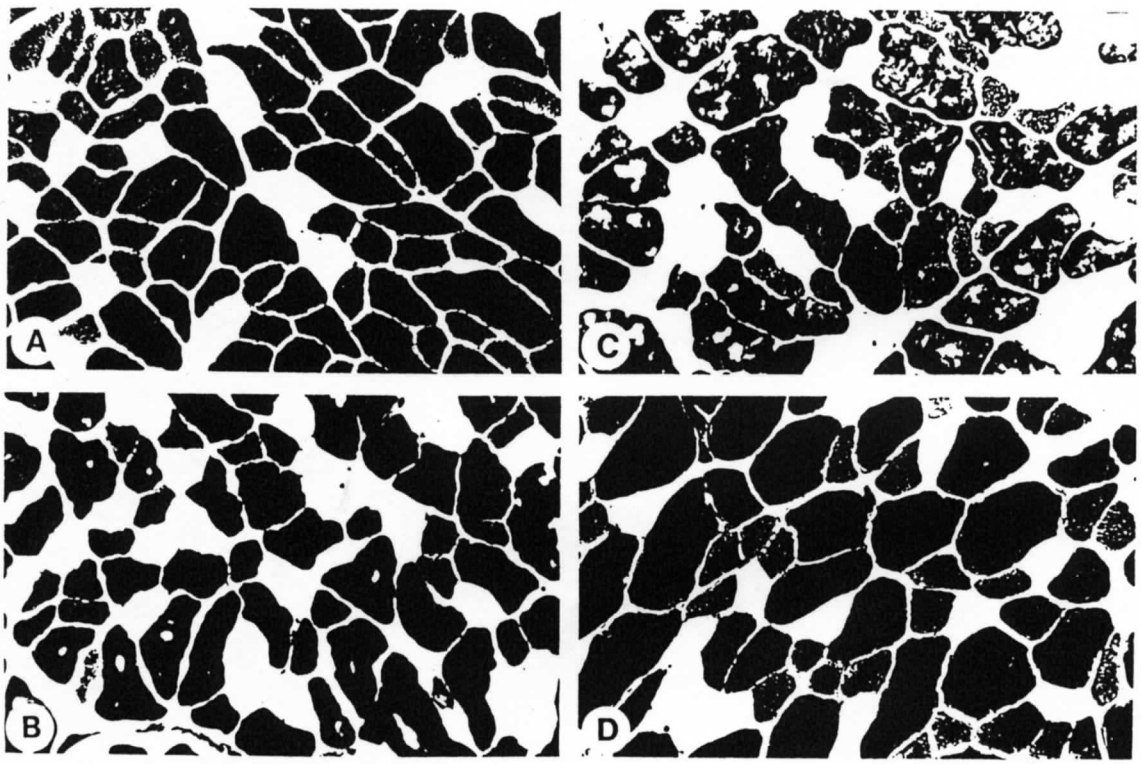

Fig. 3

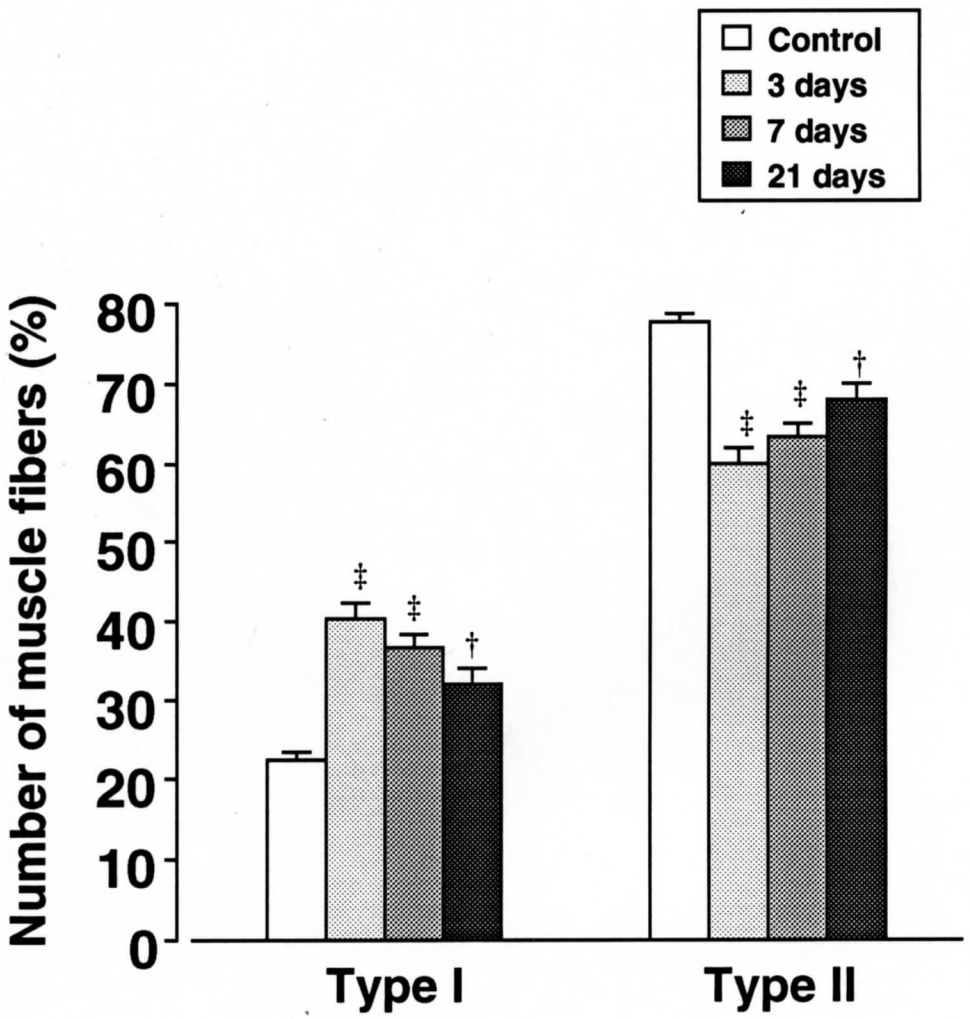
Control
3 days
7 days
21 days
Number of muscle fibers (%)
80
70
60
50
40
30
20
10
0
‡
‡
†
‡
‡
†
Type I
Type II

Fig. 4

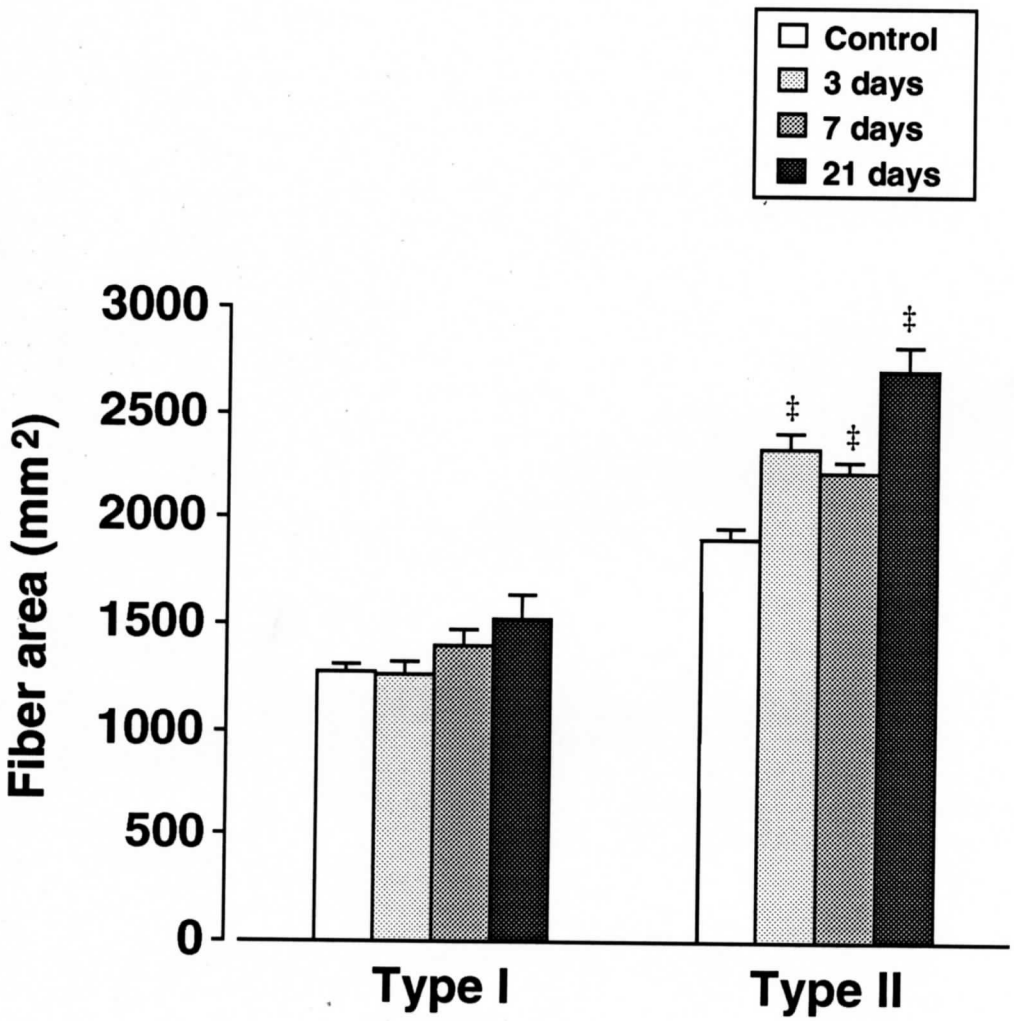
Control
3 days
7 days
21 days
Fiber area (mm²)
3000
2500
2000
1500
1000
500
0
‡
‡
‡
Type I
Type II

Fig. 5